Short Communication

Oligoclonal enzyme-linked immunosorbent assay capable of determining the major food allergen, ovomucoid, irrespective of the degree of heat denaturation

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Received 27 August 2004; accepted in revised form 9 December 2004

Key words: Allergen labeling, Enzyme-linked immunosorbent assay, Food allergy, Monoclonal antibody, Ovomucoid

Abstract

We have recently established an enzyme-linked immunosorbent assay (ELISA) for total ovomucoid determination, irrespective of the degree of its heat denaturation, by using a monoclonal antibody (mAb) 7D specific to the carbohydrate moiety of ovomucoid (Biosci Biothechnol Biochem, 68, 2490–2497, 2004). Two novel methods have been developed to improve the ELISA. First, its sensitivity was enhanced 100 times by using an oligoclonal cocktail of mAb 7D and two other mAbs with different epitopes as a second antibody. Second, it was shown that usage of denaturing reagents such as SDS and β -mercaptoethanol for extraction was acceptable for ELISA within a range of stability of a first antibody on a solid phase. Properties of the oligoclonal sandwich ELISA system thus constructed were discussed in connection with allergen labeling.

Abbreviations: ELISA – enzyme-linked immunosorbent assay; H-OM – heated OM; mAb – monoclonal antibody; N-OM – native OM; OM – ovomucoid; PBS – 10 mM phosphate-buffered saline pH 7.4

Introduction

From the viewpoint of preventing the occurrence of health hazards caused by foods containing allergens, mandatory labeling of five specified ingredients (eggs, milk, wheat, buckwheat, and peanuts) started from 2002 in Japan for the first

challenge in the world (Kanagawa and Imamura 2002). For the accomplishment and confirmation of this labeling, two types of enzyme-linked immunosorbent assays (ELISAs) using polyclonal antibodies were introduced according to the Notification #1106001 from The Japanese Ministry of Health, Labour and Welfare (Honjoh et al.

2002; Takahata and Morimatsu 2002). However, they are requested to be further improved along with scientific progress as soon as possible. Hen's egg is the material that causes food allergy most frequently (Imai and Ikura 2003) and approximately two-thirds of children diagnosed as food allergy are reactive to egg white (Sampson and Ho 1997). Ovomucoid (OM), a glycoprotein of a molecular weight of 28 kDa, is thought to be the major allergen in egg white (Bernhisel et al. 1994). Recently, we have reported three kinds of monoclonal antibodies (mAbs) and sandwich ELISAs that can recognize native and/or thermally induced denatured forms of OM (Hirose et al. 2004). Here we have established an oligoclonal sandwich ELISA system for OM by mixing these three independent mAbs, which possesses several ideal features required for the next generation of allergen detection system accepted all over the world.

Materials and methods

Preparation of OM

Native OM (N-OM) was chromatographically purified. To freshly prepared hen's egg white was added an equal volume of 0.1 M Acetate buffer pH 3.8 and the mixture was dialyzed against the same buffer overnight. After centrifugation, resulting supernatant was applied to a column of carboxymethyl-Toyopearl 650 M (TOSO, Tokyo, Japan). OM was eluted by a linear gradient of 0–0.2 M of NaCl in 20 mM Acetate buffer pH 4.6. Heated OM (H-OM) was prepared by heating 1 mg/ml of N-OM in 10 mM phosphate-buffered saline pH 7.4 (PBS) at 100 °C for 30 min.

Monoclonal antibodies

Hybridomas producing mAbs specific to OM were established by fusion of splenocytes of BALB/c mice immunized with N- or H-OM and myeloma cells (NSI/1-Ag4-1) as described previously (Narita et al. 1995). Those with interest were cloned twice and mAbs named after them were prepared from their ascitic fluids and purified on Protein G-Sepharose columns (Amersham Biosciences Corp., NJ, USA). MAb 5C and 10D are specific to N-OM, mAb 6H is specific to H-OM,

and mAb 7D is specific to carbohydrate moiety of OM. Details of their specificity were described previously (Hirose et al. 2004). MAb 5C was used in this paper as N-OM specific one because mAb 10D became less specific to N-OM. The reason was uncertain, however, Yamada et al., reported the specificity change of a mAb by the change in its carbohydrate moiety occurred during cell culture (Fujimura et al. 2000). Portions of purified mAbs were biotinylated with EZ-Link sulfo-NHS-LC-Biotin (Pierce, IL, USA).

Sandwich ELISA

Wells in an ELISA plate (Nunc, Roskilde, Denmark) were first coated commonly with 5 µg/ml of mAb 7D. After blocking with PBS containing 1% BSA, standard OM or a sample was added to the wells. Then, the amount of OM bound to a well was assayed indirectly by using alkaline phosphatase-conjugated streptavidin (Oncogene, Boston, MA, USA) via biotinylated second mAb(s), after which each sandwich ELISA system was named. Immunological and enzymatical reactions were carried out at 37 °C. At each step, wells were washed thoroughly with Tris-buffered saline pH 7.4 containing 0.05% Tween 20. Absorbance at 405 nm was measured for the enzyme reaction.

Results and discussion

Oligoclonalization of ELISA

MAb 7D is specific to carbohydrate moiety of OM. Although a sandwich ELISA is usually unable to set up by a single mAb, it was successful in the case of mAb 7D because of 4 or 5 carbohydrate chains per one molecule of OM (System 7D in Figure 1). System 7D showed an equal reactivity against N- and H-OMs, indicating the possibility to determine total OM content irrespective of the degree of heat denaturation of OM. It is a very important nature required for the allergen detection system because of the allergenicity of H-OM (Hirose et al. 2004). However, the determination limit of System 7D was rather high and about 100 ng/ml for both N- and H-OMs. It is known that ELISA constructed with polyclonal antibody usually exhibits higher

Figure 1. Oligoclonalization of sandwich ELISA specific to OM. Oligoclonal sandwich ELISAs were constructed with mAb 7D as a common solid phase antibody and 50 μ l of biotinylated second mAb(s). In System 7D was used 0.5 μ g/ml of biotinylated mAb 7D, 0.5 μ g/ml each of biotinylated mAbs 5C and 7D in System 5C· 7D, 0.2 μ g/ml of biotinylated mAb 6H and 0.5 μ g/ml of biotinylated mAbs 7D in System 6H· 7D, and 0.2 μ g/ml of biotinylated mAb 6H and 0.5 μ g/ml each of biotinylated mAbs 5C and 7D in System 5C· 6H· 7D. N-OM (open circles) and H-OM (closed circles) were used as standards and the amount of OM bound to a well was assayed by alkaline phosphatase-conjugated streptavidin and *p*-nitrophenylphosphate.

sensitivity than that with mAb. Additive effect of another biotinylated mAb to biotinylated mAb 7D was expected and we have two mAbs with different epitopes from that of mAb 7D. MAb 5C is specific to N-OM and mAb 6H is specific to H-OM. When a mixture of biotinylated mAbs 5C and 7D was used as the second antibody, sensitivity only to N-OM was improved about 30 times (System 5C· 7D in Figure 1). Contrarily by using that of biotinylated mAbs 6H and 7D, similar enhancement of sensitivity was seen only to H-OM in turn (System 6H· 7D in Figure 1). Then by using that of three biotinylated mAbs, the original sensitivity of System 7D was finally improved about 100 times for both N- and H-OMs (System 5C· 6H· 7D in Figure 1). The amounts of biotinylated mAbs used were minimum and essential to get that sensitivity. Increase of biotinylated mAb 6H was inhibitory. System 7D has a disadvantage to crossreact with two other minor proteins with OM type of carbohydrate moiety (Harvey et al. 2000; Hirose

et al. 2004). Oligoclonalization might be the help for the improvement by lowering the contribution of carbohydrate chain. We used here only mAb 7D as a solid antibody because we can determine N-and/or H-OM according to the purpose by selecting proper second antibody from biotinylated mAb 7D, 5C, 6H, or a mixture of them. Optimization of an oligoclonal ELISA should be examined in each case.

Extraction with denaturing reagents

To enhance the overall sensitivity of the assay system, it is important to improve not only determination method but also extraction method. We found that mAb 7D immobilized onto a ELISA plate was stable even in the presence of 0.1% SDS and 0.1 M β -mercaptoethanol at 37 $^{\circ}$ C for 60 min (data not shown). It has been known that proteinous samples can be usually well solu-

bilized in the condition of sample preparation for SDS-PAGE (Laemmli 1970). Then, we decided to extract OM from foods in 1% SDS and 1 M β -mercaptoethanol at 100 °C for 5 min. After cooling and diluting 20 times with PBS, OM was determined with oligoclonal System 5C·6H·7D (Table 1). There was not big difference between the values extracted with or without denaturing reagents in the case of simple or moderately processed foods. However, 6–30 fold difference were seen for foods processed under complex and/or severe conditions. Extraction with the denaturing reagents was effective even for well soluble and non-heat precipitable protein such as OM, much more for many other proteins that are difficult to be soluble in water or heat-precipitable. Moreover, if a food is proved to be not containing allergens or non-allergenic by an *in vitro* tests because of some masking effects such as modification, aggregation (Kato et al. 2001), and immune complex formation (Hirose et al. 2001), its allergenicity after eating is uncertain. Extraction with denaturing reagents might enable us to determine antigens in foods closer to an absolute quantity by removing the influence of masking factors.

There is an international movement for allergen labeling, as seen in the agreement of the Joint

FAO/WHO Codex Alimentarius Commission Session in 1999. In consideration of worldwide distribution of foods today, development of an international common method is required for the adequate determination of allergens. Oligoclonal system is suitable for the purpose because it is more stable than polyclonal system in specificity and quantity due to the nature of mAbs. In addition to the mandatory labeling, furthermore, other twenty items (abalone, squid, salmon roe, shrimp/prawn, oranges, crab, kiwifruit, beef, tree nuts, salmon, mackerel, soybeans, chicken, pork, mushrooms, peaches, yams, apples, gelatin, and banana) are recommended to be labeled as many as possible by ministerial notice in Japan. It is expected that oligoclonal system and extraction with denaturing reagents described here would throw new concepts for the construction of the relevant determination systems for the labeling of foods containing allergens.

Acknowledgement

This work was supported in part by a grant from the Japan Society for the Promotion of Science.

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Table 1. Determination of OM in foods extracted with or without denaturing reagents by the oligoclonal sandwich ELISA.

Foods	Extraction	
	Without SDS & Mer	With SDS & Mer
Raw egg	10.3	13.3
Boiled egg	11.2	11.4
Retorted egg	7.5	8.5
Kamaboko	0.080	0.054
Steamed bun	0.025	0.179
Hard biscuit	0.021	0.298
Chinese noodle	0.004	0.105

Fresh chicken egg was boiled in water for 20 min or retorted for 20 min at 120 °C. Kamaboko (a traditional Japanese food, semicylindrically shaped fish paste), steamed bun, hard biscuit, and Chinese noodle with labels of egg usage were purchased at a market. After homogenizing whole eggs and foods individually, two gram each was mixed vigorously in 38 ml of PBS with or without 1% SDS and 1 M β -mercaptoethanol (Mer). Samples extracted with denaturing reagents were boiled at 100 °C for 5 min and cooled. After centrifugation at 13,000 $\times$ g for 10 min, a portion of the supernatant was diluted 20 times with PBS and OM was determined by System 5C·6H·7D in Figure 1. Values were expressed as mg/g.

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